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CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, vol. 96, 1982, pages 119-144, Springer Verlag, Berlin, DE; C.P. HOLLENBERG: "Cloning with 2-mu DNA vectors and the expression of foreign genes in *saccharomyces cerevisiae*"

BIOLOGICAL ABSTRACTS, 1986, page 78, abstract no. 33008878; C.V. BRUSCHI: "A new system for in-vivo study of the yeast 2mu DNA plasmid", & PLASMID 1987, vol. 17, no. 1, page 78

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Description

This invention relates to genetic engineering in yeast, especially *Saccharomyces cerevisiae*.

The uptake of exogenous DNA by yeast cells and the subsequent inheritance and expression of that DNA are brought about by a process called transformation. Transformation was first described in the late 1970's, employing methods which rely upon the addition of DNA to protoplasts produced by the enzymic removal of the yeast cell wall (Hinnen et al., 1978; Beggs, 1978). More recently the transformation of intact yeast cells has been demonstrated (Hisao et al., 1983).

Yeast can be transformed by appropriate plasmids; plasmids used for this purpose are usually constructed as "shuttle vectors" which can be propagated in either *Escherichia coli* or yeast (Hinnen et al., 1978; Beggs, 1978; Struhl, et al., 1979). The inclusion of *E.coli* plasmid DNA sequences, such as pBR322 (Bolivar, 1978), facilitates the quantitative preparation of vector DNA in *E.coli*, and thus the efficient transformation of yeast.

Plasmid vectors commonly in use for yeast transformation can be divided into two types: (i) replicating vectors, that is those which are capable of mediating their own maintenance, independent of the chromosomal DNA of yeast, by virtue of the presence of a functional origin of DNA replication and (ii) integrating vectors, which rely upon recombination with the chromosomal DNA to facilitate replication and thus the continued maintenance of the recombinant DNA in the host cell. Replicating vectors can be further sub-divided into: (a) 2 μ m-based plasmid vectors in which the origin of DNA replication is derived from the endogenous 2 μ m plasmid of yeast, (b) autonomously replicating vectors (ARS) in which the "apparent" origin of replication is derived from the chromosomal DNA of yeast and (c) centromeric plasmids (CEN) which carry in addition to one of the above origins of DNA replication a sequence of yeast chromosomal DNA known to harbour a centromere.

In order to transform yeast efficiently with any of the aforementioned vectors it is necessary to impose a selection to identify those transformants which carry the recombinant DNA. This is achieved by incorporating within the vector DNA a gene with a discernible phenotype. In the case of vectors used to transform laboratory yeast, prototrophic genes, such as *LEU2*, *URA3* or *TRP1* (Hinnen et al., 1978; Beggs, 1978; Gerbaud et al., 1979), are usually used to complement auxotrophic lesions in the host. However, in order to transform brewing yeast and other industrial yeasts, which are frequently polyploid and do not display auxotrophic requirements, it is necessary to utilize a selection system based upon a dominant selectable gene. In this respect replicating 2 μ m based plasmid vectors have been described carrying genes which mediate resistance to: (i) antibiotics, for example G418 (Jimenez et al., 1980; Webster et al., 1983), hygromycin B (Gritz et al., 1983), chloramphenicol (Cohen et al., 1980; Hadfield et al., 1986), and (ii) otherwise toxic materials, for example the herbicide sulfometuron methyl (Falco et al., 1985), compactin (Rine et al., 1983) and copper (Henderson et al., 1985).

The inheritable stability of recombinant genes in yeast is dependent upon the type of yeast vector employed to facilitate transformation. The most stable of the two types of vector systems described earlier are the integrating vectors. The principles and practice of integrative yeast transformation have been described in the literature (Botstein & Davis, 1982; Winston et al., 1983; Orr-Weaver et al., 1983; Rothstein, 1983). In general, integrative transformation is relatively inefficient; closed circular integrating plasmids have been described which yield approximately 1-10 transformants per μ g of DNA (Hinnen et al., 1979; Hicks et al., 1979). However, linear DNA, with free ends located in DNA sequences homologous with yeast chromosomal DNA, transforms yeast with higher efficiency (100-1000 fold) and the transforming DNA is generally found integrated in sequences homologous to the site of cleavage (Orr-Weaver et al., 1981). Thus by cleaving the vector DNA with a suitable restriction endonuclease, it is possible to increase the efficiency of transformation and target the site of chromosomal integration. Integrative transformation is applicable to the genetic modification of brewing yeast, providing that the efficiency of transformation is sufficiently high and the target DNA sequence for integration is within a region that does not disrupt genes essential to the metabolism of the host cell. An integrating yeast vector has recently been described for brewing yeast (Yocum, 1985).

Unlike integrating vectors, which show a high degree of inheritable stability in the absence of selection, replicating vectors tend to be more unstable. The degree of inheritable stability is dependent upon the type of replicating vector used. ARS plasmids, which have a high copy number (approximately 20-50 copies per cell) (Hyman et al., 1982), tend to be the most unstable, and are lost at a frequency greater than 10% per generation (Kikuchi, 1983). However, the stability of ARS plasmids can be enhanced by their attachment of a centromere; centromeric plasmids are present at 1 or 2 copies per cell (Clarke & Carbon, 1980) and are lost at only approximately 1% per generation (Walmsley et al., 1983). Chimaeric 2 μ m based plasmids show varying degrees of inheritable stability, dependent upon both the host strain and the 2 μ m DNA sequences

present on the plasmid.

The 2 μ m plasmid is known to be nuclear in cellular location (Nelson & Fangman, 1979; Livingston & Hahne, 1979; Seligy *et al.*, 1980; Taketo *et al.*, 1980; Sigurdson *et al.*, 1981), but is inherited in a non-Mendelian fashion (Livingston, 1977). Cells without the 2 μ m plasmid (cir⁻) have been shown to arise from haploid yeast populations having an average copy number of 50 copies of the 2 μ m plasmid per cell at a rate of between 0.001% and 0.01% of the cells per generation (Futcher & Cox, 1983). A possible explanation for this low level of inheritable instability is that the plasmid provides no obvious advantage to the cell under normal growth conditions (Broach, 1981; Futcher & Cox, 1983; Sigurdson *et al.*, 1981), although small effects on growth rates have been reported for some strains harbouring the 2 μ m plasmid (Walmsley *et al.*, 1983). Analysis of different strains of *S. cerevisiae* has shown that the plasmid is present in most strains of yeast (Clark-Walker & Miklos, 1974) including brewing yeast (Tubb, 1980; Aigle *et al.*, 1984; Hinchliffe & Daubney, 1986). It thus appears that the plasmid is ubiquitous, which implies a high degree of inheritable stability in nature.

Genetic and molecular analysis of the 2 μ m plasmid has revealed a wealth of information concerning the replication and stable maintenance of the plasmid (Volkert & Broach, 1987). In essence the plasmid consists of a circular DNA molecule of 6318 base-pairs (Hartley & Donelson, 1980). It harbours a unique bidirectional origin of DNA replication (Newlon *et al.*, 1981) which is an essential component of all 2 μ m based vectors. The plasmid contains four genes, REP1, REP2, REP3 and FLP which are required for the stable maintenance of high plasmid copy number per cell (Broach & Hicks, 1980; Jayaram *et al.*, 1983). REP3 is also referred to as the STB locus. The REP1 and REP2 genes encode trans acting proteins which are believed to function in concert by interacting with the REP3 locus to ensure the stable partitioning of the plasmid at cell division (Volkert & Broach, 1987). In this respect the REP3 gene behaves as a cis acting locus which effects the stable segregation of the plasmid, and is phenotypically analogous to a chromosomal centromere (Jayaram *et al.*, 1983; Kikuchi, 1983). An important feature of the 2 μ m plasmid is the presence of two inverted DNA sequence repetitions (each 559 base-pairs in length) which separate the circular molecule into two unique regions. Intramolecular recombination between the inverted repeat sequences results in the inversion of one unique region relative to the other and the production *in vivo* of a mixed population of two structural isomers of the plasmid, designated A and B (Beggs, 1978). Recombination between the two inverted repeats is mediated by the protein product of a gene called the FLP gene, and the FLP protein is capable of mediating high frequency recombination within the inverted repeat region. This site specific recombination event is believed to provide a mechanism which ensures the amplification of plasmid copy number (Futcher, 1986; Volkert & Broach, 1986; Som *et al.*, 1988; Murray *et al.*, 1987).

Each inverted repeat sequence comprises three DNA repeat sequence sub-units (depicted as triangles in Figure 3), two adjacent sub-units being in mutually direct orientation and the third being in indirect orientation and joined to one of the other sub-units via an 8 base pair linking or spacer region. This spacer region contains a unique XbaI site and recognises and is cut at its margins by the product of the FLP gene. The adjacent sequences are of course homologous to the corresponding sequences of the other inverted repeat sequence and hence provide for accurate recombination following the said cutting. Andrews *et al.*, (1985) has found that a 74 base pair sequence including the 8 b.p. spacer region is the minimum requirement for FLP site specific recombination.

Yeast vectors based upon the replication system of the 2 μ m plasmid have been constructed by inserting heterologous DNA sequences in regions of the 2 μ m plasmid not essential to its replication (Beggs, 1981). This has resulted in two basic types of vector: (i) whole 2 μ m vectors and (ii) 2 μ m origin vectors. In the case of the former, these vectors harbour the whole 2 μ m plasmid into which various heterologous sequences have been inserted, such as E.coli plasmid DNA. These plasmids are capable of maintaining themselves at high copy number with a high degree of inheritable stability in both cir⁺ (2 μ m containing) and cir⁻ (2 μ m deficient) hosts. On the other hand 2 μ m origin vectors usually contain a minimal DNA sequence harbouring the 2 μ m origin of DNA replication and a single copy of the 599 base-pair repeat of 2 μ m; such vectors can only be maintained in cir⁺ host strains, since they require the proteins encoded by the REP1 and REP2 genes to be supplied in trans from the endogenous plasmid to ensure their 'stable' maintenance.

When a genetically modified yeast which is capable of expressing a heterologous gene to produce high levels of a commercially important polypeptide is constructed, it is usually desirable to choose a high copy number yeast vector. 2 μ m based vectors have proved very successful for use as expression plasmids and therefore frequently constitute the vector of choice (Kingsman *et al.*, 1985).

In European Patent Application 86303039.1 (Publication No. 0201239 A1 in the name of Delta Biotechnology Ltd.) a process is described for the production of heterologous proteins in brewing yeast, in which an industrial yeast strain is genetically modified to be capable of expressing a heterologous gene,

such that no expression of the said heterologous gene takes place during the primary beer fermentation, but rather yeast biomass is accumulated and the synthesis of heterologous protein is induced after the yeast has been removed from the beer. This is achieved by transforming brewing yeast with a 2 μ m based plasmid harbouring the dominant selectable marker CUP-1 and a gene encoding a modified human serum protein, N-methionyl albumin (Met-HSA), whose expression is regulated at the transcriptional level by a galactose inducible promoter. In order to maximise the yield of heterologous protein synthesis during the operation of the said process it is necessary to ensure: (i) a high copy number of the gene to be expressed (encoding for Met-HSA); (ii) a high degree of inheritable stability of the gene of interest under conditions of non-selective growth; (iii) that the recombinant genes transformed into brewing yeast must not have a deleterious effect upon the yeast and its ability to produce beer and subsequently heterologous protein; and (iv) that the recombinant genes present in yeast should, so far as possible, be restricted to the 'gene of interest' and adjacent yeast regulatory genes. The requirement (ii) is particularly important because it is both impractical and undesirable to supplement the normal growth medium of brewers' yeast, namely hopped malt extract, with toxic materials such as copper ions since this will increase process costs and have a deleterious and probably unacceptable effect upon the quality of the beer, which is the primary product of the fermentation. In connection with requirement (iv), it is desirable that the genetically modified yeast should not possess extraneous DNA sequences such as those which are derived from the bacterial portion of the recombinant plasmid.

In our application published as EP-A-251744 we have described a method for modifying yeast cells by incorporating into the endogenous 2 μ m plasmid a DNA sequence coding for a protein or peptide of interest, by making an integration vector comprising two copies of a homologous 2 μ m plasmid DNA sequence in direct orientation encompassing the DNA sequence of interest, transforming yeast with the said integration vector, and then isolating from the transformed yeast obtained cells containing the endogenous 2 μ m plasmid modified by incorporation of the DNA sequence of interest. The integration vector itself does not survive in the transformed yeast cells. The homologous 2 μ m plasmid DNA sequences may be, but usually are not, copies of the 2 μ m plasmid repeat sequence.

We have now found that a modification of the method of the said application make it possible to transform yeast cells by incorporation of a modified 2 μ m plasmid.

In the method of the present application, the plasmid vector used comprises a DNA sequence which allows propagation of the vector in bacteria encompassed between two homologous 2 μ m plasmid DNA FLP recombination sites in direct orientation, a DNA sequence coding for a protein or peptide of interest, which is preferably but not necessarily a sequence heterologous to yeast, and preferably also a selectable marker DNA sequence. The 2 μ m plasmid vector of the invention thus comprises three copies of the FLP recombination site of which one pair is in direct orientation and the other two pairs are in indirect orientation. When yeast is transformed with a plasmid vector having this construction, the DNA sequence which allows propagation of the vector in bacteria has been found to be spontaneously lost and the plasmid vector becomes a modified 2 μ m plasmid which is capable of replacing the endogenous 2 μ m plasmid in the transformed yeast. Plasmid vectors of this type are hereinafter called disintegration vectors. The yeast transformed with such vectors may contain multiple extrachromosomal copies of a modified 2 μ m plasmid containing a gene of interest but no bacterial DNA, which have been found to be stably inherited under conditions of non-selective growth.

Bruschi (13th International Conference on Yeast Genetics and Molecular Biology, Autumn 1986) disclosed that recombination in a 2 μ -based plasmid could result in the excision of bacterial DNA sequences, but suggested only that the system could be used to study structure-function relationships in the DNA molecule. Bruschi (Plasmid 1987, 17(1), p.98) is similar but mentions cloning genes. Hollenberg (Curr. Topics in Microbiol. & Immunol., 1982, 96, pp.119-144) discusses recombination in 2 μ -based plasmids. None of these documents teaches a preferred insertion site for the desired coding sequence. We have now found that a similar system can be used to prepare advantageous expression vectors which have unexpected stability, when the desired DNA coding sequence is inserted between ori and the STB gene of the 2 μ m plasmid-derived DNA.

The term "FLP recombination site" is used herein to mean any site which will allow for recombination as a result of interaction with the FLP gene product. If Andrews' finding (1985) is correct, then the FLP recombination site will generally comprise as a minimum the 74 b.p. sequence identified by him. In practice, there is no point in including more than the 599 base pairs of the whole repeat sequence.

The 2 μ m based disintegration vector of the present invention has been found to be capable of transforming both laboratory and industrial yeast. The disintegration vector is maintained at a high copy number per cell and has an extremely high degree of inheritable stability. In addition, unlike all other 2 μ m based plasmid vectors thus far described, the disintegration vector is constructed so that, upon transforma-

tion of yeast, the bacterial plasmid DNA sequences are spontaneously deleted. Thus genetically modified strains of brewing yeast can be constructed in which the 'gene of interest' incorporated in the 2 μ m plasmid is stably maintained, even under conditions of non-selective growth, at a high copy number per cell, in the absence of extraneous bacterial plasmid DNA sequences. The use of such a vector in the construction of a

5 genetically modified brewing yeast ensures that only the 'gene of interest' is stably maintained for successive generations in brewing yeast, thereby circumventing any potential deleterious effects that additional DNA sequences may have upon the technological behaviour of the yeast and/or the flavour and quality characteristics of beer produced by the yeast.

In practice the 'gene of interest' can be any recombinant gene, either homologous or heterologous to yeast. The disintegration vector can be used for example to stably integrate the Met-HSA gene in brewing yeast expressed from either a constitutive yeast promoter, for example the phosphoglycerate kinase promoter (PGK) in accordance with the method described in EP-A-147 198 or a regulated yeast promoter, for example the GAL10/CYC1 hybrid promoter as described in EP-A-201 239, or the GAL/PGK promoter as described in EP-A-258 067.

15 Additional genes which may be stably maintained by this system include the DEX1 gene of Saccharomyces diastaticus which specifies the production of an extracellular glucoamylase enzyme in brewing yeast, and the β -glucanase gene of Bacillus subtilis which specifies the production of an endo-1,2-1,4- β -glucanase in brewing yeast (Hinchliffe & Box, 1985). Such genes can be first genetically modified to control the level of gene expression and/or to ensure that the protein whose synthesis is mediated by the gene is

20 secreted by the brewing yeast.

The use of the new disintegration vector is particularly advantageous in the process described in EP-A-201 239, since, according to this process, the 'gene of interest' is regulated so that it is not expressed during the course of the beer fermentation nor under normal conditions of yeast growth, but is rather induced in a post fermentation process. Consequently high level expression of the 'gene of interest' is

25 divorced in time from the synthesis of yeast biomass by cell proliferation, and thus any adverse effects of gene expression upon plasmid stability are minimized.

Preferably the vector of the present invention is a disintegration vector (as hereinbefore defined) comprising a complete 2 μ m plasmid additionally carrying (i) a bacterial plasmid DNA sequence necessary for propagation of the vector in a bacterial host; (ii) an extra 2 μ m FLP recombination site; (iii) a DNA

30 sequence coding for a desired protein or peptide inserted between ori and the STB gene; and (iv) a selectable marker DNA sequence for yeast transformation; the said bacterial plasmid DNA sequence being present and the extra FLP recombination site being created at a restriction site in one of the two inverted repeat sequences of the 2 μ m plasmid, the said extra FLP recombination site being in direct orientation in relation to the endogenous FLP recombination site of the said one repeat sequence, and the bacterial

35 plasmid DNA sequence being sandwiched between the extra FLP recombination site and the endogenous FLP recombination site of the said one repeat sequence.

The preferred disintegration vector thus consists of a complete 2 μ m plasmid into which is inserted one or more bacterial plasmid DNA sequences and an extra copy of a 74 base-pair FLP recombination site derived from the 2 μ m plasmid. In addition the 'gene of interest', co-linear with a selectable marker for yeast transformation, e.g. CUP-1, is inserted between ori and the STB gene in the 2 μ m plasmid. The bacterial

40 plasmid DNA sequences and yeast DNA repeat are inserted, e.g. at an XbaI site, in one copy of the two inverted repeats of the whole 2 μ m plasmid. The correct orientation of the DNA repeat is essential to the function of the plasmid; the plasmid is constructed so that the bacterial plasmid sequence necessary for DNA propagation in, for example, E.coli, is sandwiched between two copies of the FLP recombination site of the 2 μ m plasmid, which are themselves in direct orientation. The configuration of DNA sequences is

45 illustrated in Figure 3 described in more detail below. This construction confines the bacterial plasmid DNA sequences to a region of DNA which, when the plasmid is transformed into yeast, is excised from the plasmid, by virtue of a site-specific recombination event between the two directly oriented DNA repeats. This site-specific recombination is mediated by the product of the FLP gene of 2 μ m whose product can

50 either be supplied by the endogenous 2 μ m plasmid of yeast, when transforming cir⁺ cells, or by the disintegration vector itself when transforming cir⁻ cells. Because the vectors of the invention may be used to cure the transformed yeast of its endogenous 2 μ m plasmids, and also because the recombination is more rapid in cir⁻ cells, it is preferable for the vector of the invention to be based on a complete 2 μ m plasmid. If however, the vector of the invention is to co-exist with the endogenous 2 μ m plasmids, then

55 genes such as REP1, REP2, REP3 and FLP need not be present on the vector, as the products of these genes can be supplied in trans; all that is necessary is an origin of replication.

As is described in more detail below, the inserted DNA carrying the bacterial sequences may carry at each end a respective portion of the repeat sequence, in which case the said DNA is inserted into an

endogenous repeat sequence such that the endogenous recombination site is effectively destroyed but two new FLP recombination sites are formed, each comprising a portion of the endogenous recombination site and a complementary portion from the inserted DNA. Alternatively, a complete FLP recombination site may be carried towards one end of the insert, which insert is then inserted adjacent to or spaced from an endogenous repeat sequence such that the bacterial DNA lies between the endogenous repeat sequence and the inserted repeat sequence. When the inserted DNA is inserted at a location spaced from the endogenous sequence, the endogenous DNA between the endogenous repeat sequence and the inserted repeat sequence will later be excised along with the bacterial DNA. Hence, if this DNA is needed, a further copy of it must be provided (preferably on the inserted DNA) on the side of the inserted repeat sequence remote from the endogenous repeat sequence.

The site within the integral 2µm plasmid at which the 'gene of interest' is inserted is chosen with a view to minimizing the effect of the insertion upon both plasmid copy number and inheritable stability. Thus it is advantageous to insert the 'gene of interest' at a site that does not interrupt the integrity of the REP1, REP2, REP3 and FLP genes, particularly if the plasmid is intended for use in the transformation of a *cir*⁺ host strain of yeast. The region between *ori* and STB gene meets these criteria.

One advantageous characteristic of the disintegration vector is that, when it is introduced into *cir*⁺ yeast strains, because it possesses an integral 2µm plasmid, it is capable of curing the endogenous 2µm plasmid, either during or following the excision of the bacterial plasmid sequences. An analogous situation has recently been reported for whole 2µm vectors transformed into *cir*⁺ host strains of yeast (Harford & Peters, 1987). Thus the disintegration vector can also be used to cure the endogenous 2µm plasmid from strains of yeast.

In the accompanying drawings,

Figure 1 shows plasmid pBA112 (Andrews, et al., 1985). The thin line represents DNA sequences derived from the bacterial plasmid pUC9; the open bar represents the 74 base-pair DNA fragment containing the FLP recombination site; the triangles indicate the orientation of the three internal DNA repeats within each FLP recombination site (Andrews, et al., (1985);

Figure 2 shows plasmid pSAC112. Plasmid pSAC112 is identical to pBA112 with the exception that the BamHI, PstI and HindIII sites have been deleted;

Figure 3 shows plasmid pSAC3. The thick line represents DNA sequences from the bacterial plasmid pUC9; the open bars represent the 74 base-pair DNA fragment containing the FLP recombination site; the thin line represents 2µm plasmid DNA sequences; the triangles indicate the orientation of the three internal DNA repeats in each FLP recombination site;

Figure 4 shows plasmid pSAC3U1. Designations are as for Figure 3;

Figure 5 is a plasmid map of pSAC3U2. Designations are as for Figure 3;

Figure 6 is a plasmid map of pSAC300. Designations are as for Figure 3;

Figure 7 is a plasmid map of pSAC310. Designations are as for Figure 3;

Figure 8 is a plasmid map of pSAC3C1. Designations are as for Figure 3;

Figure 9 is based on a photograph showing the growth of haploid yeast strains and illustrates the co-inheritance of the URA3 and bacterial bla gene; and

Figure 10 is an autoradiograph of total yeast DNA probed with ³²P labelled pSAC3 DNA.

The following Examples illustrate the invention.

EXAMPLE I

Construction of Plasmids

Plasmid pSAC112 (Figure 2) was constructed by digesting plasmid pBA112 (Figure 1, Andrews, et al., 1985) with the restriction endonucleases BamHI and HindIII simultaneously. Linear plasmid DNA was treated with DNA polymerase I (Klenow) in the presence of 0.3mM dNTP's (dATP, dTTP, dCTP and dGTP) for 10 minutes at 37°C. DNA was extracted with phenol:chloroform, ethanol-precipitated and incubated overnight at 15°C in the presence of T4 DNA ligase. Ligated DNA was transformed into *E.coli* strain MC1061 (Casadaban and Cohen, 1980); plasmid pSAC112 was isolated from the resultant transformants following identification and characterisation by the method of Birnboim and Doly (1980).

Plasmid pSAC3 (Figure 3) was constructed by the following procedure. Yeast 2µm plasmid DNA was isolated from strain DRI9 as described by Guerineau, et al., (1974). Purified 2µm plasmid DNA was partially digested with the restriction endonuclease XbaI as described by Maniatis et al., (1982), and ligated with XbaI cleaved pSAC112. Ligated DNA was transformed into *E.coli* strain AG1 (obtained from NBL Enzymes Ltd., Cramlington, England.). The resultant ampicillin-resistant transformants were screened for homology to

2µm plasmid DNA following colony hybridization (Grunstein and Hogness, 1975) to ³²P labelled 2.2 kilo base-pair EcoRI fragment from plasmid pYF92 (Storms, R.K. et al., 1979). Colonies showing homology with the 2µm specific DNA probe were isolated and their plasmid DNA characterized by restriction endonuclease mapping procedures. Plasmid pSAC3 was thus obtained.

5 Plasmids pSAC3U1 (Figure 4) and pSAC3U2 (Figure 5) were constructed by cleaving plasmid pSAC3 with the restriction endonuclease PstI. Linear DNA was rendered flush-ended by treatment with T4 DNA polymerase in the presence of 0.3mM dNTP's (dATP, dTTP, dCTP and dGTP) for 10 minutes at 37°C. DNA was extracted with phenol: chloroform and ethanol-precipitated prior to ligation. Plasmid pJDB110 (Beggs, 1981) was digested with the restriction endonuclease HindIII and the DNA fragments were
10 subjected to agarose gel electrophoresis on a 1% gel. A 1.1 kilo-base-pair DNA fragment, harbouring the URA3 gene of yeast, was isolated from the gel (Maniatis, et al., 1982) and treated with DNA polymerase I (Klenow) in the presence of 0.3mM dNTP's (dATP, dTTP, dCTP and dGTP). The 1.1 kilo-base-pair HindIII fragment was extracted with phenol:chloroform, ethanol-precipitated and blunt-end ligated with linear pSAC3 DNA prepared as described above. Ligated DNA was transformed into E.coli strain AG1. The resultant
15 ampicillin resistant transformants were screened for homology with the URA3 gene following colony hybridization (Grunstein and Hogness, 1975) to a ³²P labelled 1.1 kilo-base-pair HindIII fragment purified from plasmid pJDB110. Plasmids pSAC3U1 (Figure 4) and pSAC3U2 (Figure 5) were isolated from the colonies which showed homology to the URA3 gene probe. The 1.1 kilo-base-pair HindIII DNA fragment carrying the URA3 gene was also blunt-end ligated into the unique EagI and SnaBI sites of pSAC3 to give
20 plasmids designated pSAC300 (Figure 6) and pSAC310 (Figure 7) respectively.

Plasmid pSAC3C1 (Figure 8) was constructed by blunt end ligating a 694 base-pair XbaI-KpnI DNA fragment, carrying the CUP1 gene from plasmid pET13:1 (Henderson et al., 1985) into the unique PstI site of pSAC3.

25 Transformation of Yeast with Plasmids pSAC3U1 and pSAC3U2

The disintegration vectors pSAC3U1 (Figure 4) and pSAC3U2 (Figure 5) were constructed so that they each contain the selectable yeast gene, URA3, inserted at the unique PstI site of 2µm B form. In addition, each plasmid harbours DNA sequences derived from the bacterial plasmid pUC9 flanked to two copies of
30 the FLP recombination site located in direct orientation. The position of the pUC9 DNA is such that FLP mediated recombination between these two directly orientated FLP recombination sites resulted in the excision of the bacterial plasmid DNA upon transformation of yeast. Cir⁺ and cir⁻ derivatives of the haploid yeast strain S150-2B (Cashmore, et al., 1986) were transformed to uracil prototrophy with plasmids pSAC3U1 and pSAC3U2, according to the method of Ito (1983). URA⁺ transformants were screened for the
35 co-inheritance of the bacterial bla gene, which encodes the β-lactam specific enzyme β-lactamase in yeast, by the method of Chevalier and Aigle (1979). The results presented in Figure 9 show that both plasmids segregate the bla gene from the URA⁺ gene in all transformants of the cir⁻ strain, indicating deletion of the bacterial DNA sequences from the plasmids upon yeast transformation. However, the majority of URA⁺ transformants of the cir⁺ strain were observed to co-inherit the bla gene (15 out of 20 and 18 out of 20 for
40 pSAC3U1 and pSAC3U2, respectively). These data suggest that the efficiency of plasmid disintegration, i.e. FLP mediated excision of the bacterial plasmid DNA sequences, is greater upon transformation of a cir⁻ strain than a cir⁺.

Molecular Analysis of Transformants

45 In order to determine whether the URA⁺ transformants which had segregated the bla gene (i.e. β-lactamase negative clones, bla⁻) had indeed lost the bla gene and adjacent bacterial plasmid DNA sequences, yeast DNA was analysed. Two URA⁺ bla⁻ transformants of the cir⁺ and cir⁻ strains transformed with pSAC3U1 and pSAC3U2 were grown on selective minimal medium lacking uracil and total DNA was
50 extracted by the following procedure. Actively growing cells were harvested and resuspended in 5ml 1M sorbitol, 0.025M ethylenediamine-tetracetic acid (EDTA) pH8.0, 8mg/ml dithiothreitol at 28°C for 15 minutes. Cells were harvested and resuspended in 5ml 1.2M sorbitol, 0.1M sodium citrate, 0.01M EDTA pH5.8, 0.025ul/ml zymolyase (Kirin Brewery Co. Ltd.) at 28°C until protoplasts were obtained. Protoplasts were washed three times in 1.2M sorbitol prior to resuspending in 1ml 3% sarkosyl, 0.5M tris/HCl pH7.5,
55 0.2M EDTA, 100ul/ml proteinase K at 55°C for 60 minutes. DNA preparations were extracted with chloroform:iso-propanol, phenol, chloroform and ether prior to dialysis against 10mM tris/HCl 1mM EDTA pH8. Total yeast DNA was digested with the restriction endonucleases EcoRI, XbaI and PstI and DNA fragments were separated by agarose electrophoresis. Following Southern transfer (Maniatis, et al., 1982)

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total yeast DNA was hybridized to ³²P labelled pSAC3 DNA. The results are presented in Figure 10 which is an autoradiograph of total yeast DNA probed with ³²P labelled pSAC3 DNA. DNA was isolated from S150-2B cir⁺ strains transformed with plasmids pSAC 3U1 and pSAC 3U2. Two independent transformants of each strain/plasmid combination designated A and B were analysed. DNA was digested with restriction endonuclease as follows: XbaI, tracks 1-4 and 21-24; PstI, tracks 5-12; EcoRI, tracks 13-20.

	<u>Track</u>	<u>Plasmid</u>	<u>Cir⁺/cir^o</u>	<u>Isolate (A/B)</u>
10	6, 14, 22	pSAC3U1	cir ⁺	A
	8, 16, 24	pSAC3U1	cir ⁺	B
15	5, 13, 21	pSAC3U1	cir ^o	A
	7, 15, 23	pSAC3U1	cir ^o	B
20	2, 10, 18	pSAC3U2	cir ⁺	A
	4, 12, 20	pSAC3U2	cir ⁺	B
25	1, 9, 17	pSAC3U2	cir ^o	A
	3, 11, 19	pSAC3U2	cir ^o	B

Based upon the known restriction sites present in the endogenous 2μm plasmid of yeast (Hartley and Donelson, 1980) and recombinant plasmids pSAC3U1 and pSAC3U2, one can predict the hybridization pattern to plasmid pSAC3. The predicted hybridization pattern is presented in Table 1.

TABLE 1

Expected hybridization pattern of S150-2B *cir*⁺ and *cir*⁻ derivatives, transformed with pSAC3U1 and pSAC3U2 to pSAC3

Plasmid DNA	Restriction Endonuclease Fragments (kilobase pairs)		
	<u>EcoRI</u>	<u>XbaI</u>	<u>PstI</u>
2 μ m (endogenous)	4.1	3.2	6.3
	3.9	3.1	
	2.4		
	2.2		
pSAC3U1 and	5.3	4.3	10.2
pSAC3U2 (intact)	4.1	3.2	
	0.72	2.8	
pSAC3U1 and	(5.0)	4.3	7.4
pSAC3U2 (disinte-	4.1	3.2	
grated)	3.3		
	(2.4)		

The numbers in parenthesis will arise if the disintegrated plasmids have undergone FLP mediated interconversion.

If one compares the result of the hybridization (Figure 10) with those expected (Table 1) it can be seen that in each transformant the recombinant plasmid has undergone a deletion consistent with the excision of the bacterial plasmid DNA sequences, contained within the directly oriented FLP recombination sites. In addition, in the case of the transformants designated pSAC3U2/B the endogenous 2 μ m plasmid of strain S150-2B is no longer present. This implies that transformation of a *cir*⁺ strain with plasmid pSAC3U2 results in curing of the endogenous 2 μ m plasmid.

Additional evidence that plasmids pSAC3U1 and pSAC3U2 undergo an excision of the bacterial plasmid DNA upon transformation of yeast was obtained by hybridizing the aforementioned DNA preparations to ³²P labelled pUC9 DNA (Vieira and Messing, 1982). *URA*⁺ *bla*⁻ transformants did not hybridize to this DNA probe.

Plasmids pSAC300, pSAC310 and pSAC3C1 Disintegrate upon Yeast Transformation

The *URA*⁺ plasmids pSAC300 and pSAC310 were used to transform the *cir*⁺ and *cir*⁻ derivatives of S150-2B and the *URA* and *bla* phenotypes of the resultant transformants were determined. In all cases the disintegrated phenotype was observed; thus pSAC300 and pSAC310 are capable of excising the bacterial vector DNA upon yeast transformation. In this respect it was observed that plasmid pSAC300 gave rise to a significantly higher proportion of *bla*⁻ transformants of the *cir*⁺ derivative of S150-2B. The explanation for this is unknown. However it is possible that the disruption of the *EagI* site by the insertion of the *URA3* gene in pSAC300 may have interfered with the expression of the adjacent FLP gene, resulting in over expression of the FLP recombinase.

Plasmid pSAC3C1 was designed to be used in the transformation of copper sensitive industrial yeast and in particular brewing yeast. Thus pSAC3C1 was transformed into a proprietary strain of Bass lager

yeast designated BB11.0, as described by Hinchliffe and Daubney (1986). Copper resistant transformants were then checked for the presence of the *bla* phenotype by the β -lactamase plate assay. Approximately 18% of the transformants tested were *bla*⁻ copper resistant, indicative of the *in vivo* disintegration of plasmid pSAC3C1 in the brewing yeast host.

The *in vivo* disintegration of plasmids pSAC300, pSAC310 and pSAC3C1 was subsequently confirmed following a full molecular characterization of the appropriate host strains possessing the disintegrated phenotype. Thus when total yeast DNA was hybridized to ³²P-pUC9 DNA as described previously no homology could be detected in the *bla*⁻ derivatives.

Plasmid Stability of the 'Disintegrated' Transformants

The inheritable stability of the URA⁺ phenotype in the *cir*⁺ and *cir*⁻ strains of S150-2B harbouring the disintegrated derivatives of pSAC3U1 and pSAC3U2, pSAC300 and pSAC310 was determined by growing the yeast non-selectively in YPD containing 2% w/v glucose, plating on the same medium and replica plating to minimal medium lacking uracil. The percent plasmid loss per generation was calculated and is presented in Table 2.

TABLE 2
Percent Plasmid Loss per Generation

Plasmid Derivative (Disintegrated Vector)	Percent Plasmid Loss per Generation	
	S150-2B <i>cir</i> ⁺	S150-2B <i>cir</i> ⁻
pSAC3U1	0.22	0.19
pSAC3U2	0.31	0.14
pSAC300	2.5	-
pSAC310	0	0.89

It can be seen from the result presented in Table 2 that all the 'disintegrated' derivatives are unstable in both the *cir*⁺ and *cir*⁻ derivatives of S150-2B. However, the level of instability observed for pSAC3U1, pSAC3U2, and pSAC310 in particular is at least one order of magnitude lower than that observed for other URA⁺ 2 μ m based recombinant vectors in S150-2B (Cashmore, et al., 1986).

It is interesting to note that the insertion of the URA3 gene into the unique *Eag*I site of the 2 μ m portion of pSAC3 results in a disintegrated derivative which is considerably less stable than those disintegrants derived from pSAC3U1, pSAC3U2 and pSAC310. It is apparent therefore that the site of insertion of the selectable marker can have a profound effect upon the stability of the resultant disintegrated derivative. In this respect it is clear that the unique *Sna*BI and *Pst*I sites of 2 μ m form suitable loci for the introduction of recombinant genes, since plasmid stability is not adversely affected by such insertions.

Plasmid Stability of 'Disintegrated' Transformants of Brewing Yeast

Disintegrated derivatives of pSAC3C1 transformants of BB11.0 were also analysed for the stability of the copper resistant phenotype. Plasmid stability experiments were performed as described previously and resulted in an estimate of 0.014% plasmid loss per generation, under non-selective growth conditions. It is apparent from this result that the disintegrated derivatives of pSAC3C1 are extremely stable in the brewing

yeast strain BB11.0, possessing a degree of stability hitherto unobserved for a recombinant 2 μ m based yeast vector.

Disintegration Vectors can be used to stably Maintain "Genes of Interest" in Yeast

5

Plasmid pSAC3 carries a unique PstI site and a unique SnaBI site into either of which DNA sequences can be inserted as described above, without adversely affecting the phenotype stability of the resultant disintegrated derivative of the plasmid in yeast. These sites can be used as loci for the introduction of "genes of interest" for example the DEX-1 gene of *S. diastaticus* and the human serum albumin gene expressed from a yeast promoter. Using known methods it is possible to insert such genes into these unique loci together with an appropriate selectable marker for yeast transformation. Alternatively, plasmids pSAC3U1, pSAC3U2, pSAC310 and pSAC3C1 can be used as recipients for insertion of an appropriate 'gene of interest'. In this respect pSAC3U1, pSAC3U2 and pSAC310 harbour a unique SmaI site located in the 3' non-translated region of the URA3 gene (Rose *et al.*, 1984). This SmaI site can be used as a locus for the insertion of an appropriate 'gene of interest'.

15

The desirability of using the SnaBI site to insert a gene of interest, either directly or indirectly (for example when the URA3 gene is inserted and then a gene of interest is inserted into the SmaI site thereof) is independent of the disintegration of the vector, i.e. the loss of the bacterial DNA sequences, and forms another aspect of the invention. Generally speaking, one would wish to prevent transcription continuing from the inserted gene(s) into the endogenous 2 μ m regions, particularly into the so-called STB region which is on the side of the SnaBI site remote from the yeast origin of replication (*ori*). Thus, preferably, the inserted sequence comprises (a) a gene of interest, (b) a promoter therefor on the side thereof adjacent *ori* and (c) a 3' transcription terminator downstream of the gene of interest and between that gene and the STB region.

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Claims

1. A 2 μ m plasmid vector comprising
 - 40 (a) a bacterial DNA sequence,
 - (b) yeast 2 μ m plasmid-derived DNA comprising three FLP recombination sites, an origin of replication (ori) and an STB gene and
 - (c) a DNA sequence coding for a protein or peptide of interest,
 one pair of said FLP recombination sites being in direct orientation and the other two pairs being in
 45 indirect orientation,
 the said bacterial sequence being located between the said FLP recombination sites which are in direct orientation such that, when yeast is transformed with the said vector, the said bacterial sequence is eliminated,
 the DNA sequence which codes for a protein or peptide of interest not being located between the said
 50 sites which are in direct orientation, and
 the said DNA sequence which codes for a protein or peptide of interest further being located between ori and the STB gene in the 2 μ m plasmid-derived DNA such that transcription of the said DNA coding sequence does not continue into ori or STB.
- 55 2. A 2 μ m plasmid vector according to Claim 1 which also comprises a selectable marker DNA sequence.
3. A 2 μ m plasmid vector according to Claim 1 or 2 wherein the bacterial sequence comprises a sequence allowing replication of the vector in a bacterium.

4. A 2 μ m plasmid vector according to Claim 3 wherein all bacterial plasmid DNA sequences are located between the said sites in direct orientation.
5. A 2 μ m plasmid vector according to any of Claims 1 to 4 in which the DNA sequence coding for a protein or peptide of interest is heterologous to yeast.
6. A 2 μ m plasmid vector according to Claim 5 in which the DNA sequence coding for a protein or peptide of interest is a DNA sequence coding for HSA fused at its 5' terminus to a gene promoter which functions in yeast via a secretion leader sequence which functions in yeast and at its 3' terminus to a transcription termination signal which functions in yeast.
7. A 2 μ m plasmid vector according to Claim 6 in which the DNA sequence coding for a protein or peptide of interest is the MET-HSA gene fused at its 5' terminus to the GAL/CYC1 or GAL/PGK hybrid promoter, and at its 3' terminus to a transcription termination signal which functions in yeast.
8. A 2 μ m plasmid vector according to any of Claims 1 to 4 in which the DNA sequence coding for a protein or peptide of interest is the *Saccharomyces cerevisiae* var. *diastaticus* DEX-1 gene or a DNA sequence coding for the β -glucanase of *Bacillus subtilis* fused at its 5' terminus to a gene promoter which functions in yeast via a secretion leader sequence which functions in yeast and at its 3' terminus to a transcription termination signal which functions in yeast.
9. A 2 μ m plasmid vector according to Claim 1 having substantially the configuration of pSAC3 as shown in Figure 3 of the accompanying drawings.
10. A 2 μ m plasmid vector according to any of Claims 1 to 8 and carrying a gene of interest inserted directly or indirectly at the *Sna*BI site.
11. A process for preparing a 2 μ plasmid vector according to any one of the preceding claims comprising: inserting into a complete 2 μ plasmid containing an origin of replication (ori) and an STB gene (i) a DNA sequence for selecting yeast transformants, (ii) a DNA sequence encoding a protein or peptide of interest and (iii) an insert comprising (a) bacterial plasmid DNA to allow propagation of the vector in bacteria and (b) the elements of an FLP recombination site such that an extra FLP recombination site is created in the vector and the said bacterial DNA is sandwiched between two FLP recombination sites in mutually direct orientation, the said DNA sequence which encodes a protein or peptide of interest being inserted between ori and the STB gene of the 2 μ m plasmid-derived DNA, such that transcription of the said DNA sequence does not continue through ori or STB.
12. A process according to Claim 11 wherein the insert is inserted at the unique *Xba*I site of an endogenous FLP recombination site, one end of the insert carries one portion of a 2 μ m repeat sequence and the other end of the insert carries the remainder of the 2 μ m repeat sequence.
13. A yeast cell containing a 2 μ m plasmid having an origin of replication (ori) and an STB gene and comprising a non-bacterial DNA sequence encoding a protein or peptide of interest which is heterologous to yeast, the plasmid comprising no bacterial DNA which has been transformed into the said cell, the said DNA sequence which encodes the protein or peptide of interest being located between ori and the STB gene, such that transcription of the said DNA sequence does not continue through ori or STB.
14. A yeast cell derived from a cell according to Claim 13 by reproduction of that cell and by reproduction of its progeny, and containing a plasmid comprising a DNA sequence encoding a protein or peptide of interest which is heterologous to yeast, the plasmid comprising no bacterial DNA.
15. Brewing yeast or laboratory yeast transformed with a 2 μ m plasmid vector as claimed in any of Claims 1 to 10 or 14.
16. A process for preparing a protein or peptide of interest comprising fermenting a yeast according to Claim 13, 14 or 15 and separating the protein or peptide of interest.
17. A 2 μ m yeast plasmid vector carrying a gene of interest inserted directly or indirectly at the *Sna*B1 site.

18. A 2µm plasmid vector comprising a bacterial DNA sequence, three FLP recombination sites, of which one pair of sites is in direct orientation and the other two pairs are in indirect orientation, and a DNA sequence coding for a protein or peptide of interest, the said bacterial sequence being located between the said sites which are in direct orientation such that, when yeast is transformed with the said vector, the said bacterial sequence is eliminated, and the DNA sequence coding for a protein or peptide of interest not being located between the said sites which are in direct orientation, the said vector carrying a gene of interest inserted directly or indirectly at the *Sna*BI site.

Patentansprüche

1. 2µ-Plasmid-Vektor, enthaltend

(a) eine bakterielle DNA-Sequenz,

(b) aus dem 2µ-Plasmid der Hefe stammende DNA mit drei FLP-Rekombinationsorten, einem Replikationsursprung (ori) und einem STB-Gen sowie

(c) eine DNA-Sequenz, die für ein interessierendes Protein oder Peptid codiert, wobei

ein Paar der genannten FLP-Rekombinationsorte in direkter Orientierung vorliegt und die beiden anderen Paare in indirekter Orientierung vorliegen,

die genannte bakterielle Sequenz zwischen den genannten FLP-Rekombinationsorten angeordnet ist, die in direkter Orientierung vorliegen, so daß dann, wenn Hefe mit dem Vektor transformiert wird, diese bakterielle Sequenz eliminiert wird,

die DNA-Sequenz, die für ein interessierendes Protein oder Peptid codiert, nicht zwischen den Orten angeordnet ist, die in direkter Orientierung vorliegen, und

die DNA-Sequenz, die für ein interessierendes Protein oder Peptid codiert, weiterhin zwischen ori und dem STB-Gen in der aus dem 2µ-Plasmid der Hefe stammenden DNA so angeordnet ist, daß die Transkription dieser codierenden DNA-Sequenz sich nicht in ori oder STB erstreckt.

2. 2µ-Plasmid-Vektor nach Anspruch 1, der außerdem eine selektionierbare Marker-DNA-Sequenz aufweist.

3. 2µ-Plasmid-Vektor nach Anspruch 1 oder 2, worin die bakterielle Sequenz eine Sequenz umfaßt, die die Replikation des Vektors in einem Bakterium ermöglicht.

4. 2µ-Plasmid-Vektor nach Anspruch 3, worin alle bakteriellen Plasmid-DNA-Sequenzen zwischen den in direkter Orientierung vorliegenden Orten angeordnet sind.

5. 2µ-Plasmid-Vektor nach einem der Ansprüche 1 bis 4, worin die für ein interessierendes Protein oder Peptid codierende DNA-Sequenz zu Hefe heterolog ist.

6. 2µ-Plasmid-Vektor nach Anspruch 5, worin die für ein interessierendes Protein oder Peptid codierende DNA-Sequenz eine DNA-Sequenz ist, die für HSA codiert, das an seinem 5'-Terminus mit einem Genpromotor, der in Hefe über eine in Hefe wirksame Sekretions-Leader-Sequenz wirkt, und an seinem 3'-Terminus mit einem in Hefe wirksamen Transkriptions-Terminations-Signal fusioniert ist.

7. 2µ-Plasmid-Vektor nach Anspruch 6, worin die für ein interessierendes Protein oder Peptid codierende DNA-Sequenz das MET-HSA-Gen ist, das an seinem 5'-Terminus mit dem GAL/CYC1- oder GAL/PGK-Hybridpromotor und an seinem 3'-Terminus mit einem in Hefe wirksamen Transkriptions-Terminations-Signal fusioniert ist.

8. 2µ-Plasmid-Vektor nach einem der Ansprüche 1 bis 4, worin die für ein interessierendes Protein oder Peptid codierende DNA-Sequenz das *Saccharomyces cerevisiae* var. *diastaticus* DEX-1-Gen oder eine DNA-Sequenz ist, die für die β-Glucanase von *Bacillus subtilis* codiert und an ihrem 5'-Terminus mit einem Genpromotor, der in Hefe über eine in Hefe wirksame Sekretions-Leader-Sequenz wirkt, und an ihrem 3'-Terminus mit einem in Hefe wirksamen Transkriptions-Terminations-Signal fusioniert ist.

9. 2µ-Plasmid-Vektor nach Anspruch 1, der im wesentlichen die Konfiguration des in Figur 3 der beigefügten Zeichnungen gezeigten pSAC3 aufweist.

10. 2 μ -Plasmid-Vektor nach einem der Ansprüche 1 bis 8, der ein interessierendes Gen trägt, das direkt oder indirekt an der SnaBI-Schnittstelle inseriert ist.
 11. Verfahren zum Herstellen eines 2 μ -Plasmid-Vektors nach einem der voranstehenden Ansprüche,
5 umfassend: das Insertieren
 - (i) einer DNA-Sequenz zum Selektionieren von Hefe-Transformanten,
 - (ii) einer DNA-Sequenz, die für ein interessierendes Protein oder Peptid codiert und
 - (iii) eines Inserts, das
 - 10 (a) bakterielle plasmidische DNA, um die Vermehrung des Vektors in Bakterien zu ermöglichen, und
 - (b) die Elemente eines FLP-Rekombinationsortesenthält,in ein vollständiges 2 μ -Plasmid, das einen Replikationsursprung (ori) und ein STB-Gen enthält, so daß ein zusätzlicher FLP-Rekombinationsort im Vektor geschaffen wird und die bakterielle DNA zwischen
15 zwei FLP-Rekombinationsorten in wechselseitig direkter Orientierung eingepaßt wird, wobei die DNA-Sequenz, die für ein interessierendes Protein oder Peptid codiert, zwischen ori und das STB-Gen der aus dem 2 μ -Plasmid stammenden DNA inseriert wird, und zwar derart, daß sich die Transkription der DNA-Sequenz nicht durch ori oder STB fortsetzt.
 12. Verfahren nach Anspruch 11, worin das Insert an der einzigen XbaI-Schnittstelle eines endogenen FLP-Rekombinationsortes inseriert wird, wobei ein Ende des Inserts einen Teil einer 2 μ -Repetition und das andere Ende des Inserts den Rest der 2 μ -Repetition trägt.
 13. Hefezelle, enthaltend ein 2 μ -Plasmid mit einem Replikationsursprung (ori) und einem STB-Gen und
25 ferner enthaltend eine nicht-bakterielle DNA-Sequenz, die für ein interessierendes Protein oder Peptid codiert, welches heterolog zu Hefe ist, wobei das Plasmid keine bakterielle DNA enthält, die in diese Zelle transformiert worden ist, und wobei die für ein interessierendes Protein oder Peptid codierende DNA-Sequenz zwischen ori und dem STB-Gen so angeordnet ist, daß sich die Transkription der DNA-Sequenz nicht durch ori oder STB fortsetzt.
 14. Hefezelle, die von einer Zelle nach Anspruch 13 abstammt, und zwar durch Reproduktion dieser Zelle und durch Reproduktion ihrer Nachkommen, enthaltend ein Plasmid, das eine für ein interessierendes, zu Hefe heterologes Protein oder Peptid codierende DNA-Sequenz umfaßt, wobei das Plasmid keine
35 bakterielle DNA enthält.
 15. Brauhefe oder Laborhefe, die mit einem in einem der Ansprüche 1 bis 10 oder 14 beanspruchten 2 μ -Plasmid-Vektor transformiert ist.
 16. Verfahren zum Herstellen eines interessierenden Proteins oder Peptids, umfassend das Fermentieren
40 einer Hefe gemäß Anspruch 13, 14 oder 15 und das Abtrennen des interessierenden Proteins oder Gens.
 17. 2 μ -Hefepiasmid-Vektor, der ein interessierendes Gen direkt oder indirekt an der SnaBI-Schnittstelle inseriert trägt.
 18. 2 μ -Plasmid-Vektor, enthaltend eine bakterielle DNA-Sequenz, drei FLP-Rekombinationsorte, wobei sich
45 ein Paar dieser Orte in direkter Orientierung befindet und die anderen beiden Paare in indirekter Orientierung befinden, und eine für ein interessierendes Protein oder Peptid codierende DNA-Sequenz, wobei die bakterielle Sequenz so zwischen den genannten Orten angeordnet ist, die sich in direkter Orientierung befinden, daß dann, wenn Hefe mit diesem Vektor transformiert wird, die bakterielle
50 Sequenz eliminiert wird, und wobei die für ein interessierendes Protein oder Peptid codierende DNA-Sequenz nicht zwischen den genannten Orten angeordnet ist, die sich in direkter Orientierung befinden, wobei der Vektor ein interessierendes Gen direkt oder indirekt an der SnaBI-Schnittstelle inseriert trägt.
- 55 **Revendications**
1. Vecteur plasmidique 2 μ m comprenant:
 - (a) une séquence d'ADN bactérien,

(b) un ADN de levure dérivé d'un plasmide 2 μ m comprenant trois sites de recombinaison FLP, une origine de réplication (ori) et un gène STB, et,

(c) une séquence d'ADN codant pour une protéine ou un peptide d'intérêt,

une paire desdits sites de recombinaison FLP étant en orientation directe et les deux autres paires étant en orientation indirecte,

ladite séquence bactérienne étant située entre lesdits sites de recombinaison FLP qui sont en orientation directe de telle sorte que, quand la levure est transformée par ledit vecteur, ladite séquence bactérienne se trouve éliminée,

la séquence d'ADN qui code pour une protéine ou un peptide d'intérêt n'étant pas située entre lesdits sites qui sont en orientation directe, et

ladite séquence d'ADN qui code pour une protéine ou un peptide d'intérêt étant en outre située entre ori et le gène STB dans l'ADN dérivé d'un plasmide 2 μ m de telle sorte que la transcription de ladite séquence codante d'ADN ne se continue pas dans ori ou STB.

2. Vecteur plasmidique 2 μ m selon la revendication 1, qui comprend aussi une séquence d'ADN marqueur sélectionnable.

3. Vecteur plasmidique 2 μ m selon la revendication 1 ou 2, dans lequel la séquence bactérienne comprend une séquence autorisant la réplication du vecteur dans une bactérie.

4. Vecteur plasmidique 2 μ m selon la revendication 3, dans lequel toutes les séquences d'ADN de plasmide bactérien sont situées entre lesdits sites en orientation directe.

5. Vecteur plasmidique 2 μ m selon l'une quelconque des revendications 1 à 4, dans lequel la séquence d'ADN codant pour une protéine ou un peptide intéressant est hétérologue à la levure.

6. Vecteur plasmidique 2 μ m selon la revendication 5, dans lequel la séquence d'ADN codant pour une protéine ou un peptide d'intérêt est une séquence d'ADN codant pour HSA fusionné à son extrémité 5' avec un promoteur de gène qui agit dans la levure par l'intermédiaire d'une séquence de contrôle de sécrétion qui fonctionne dans la levure, et à son extrémité 3' avec un signal de terminaison de transcription qui fonctionne dans la levure.

7. Vecteur plasmidique 2 μ m selon la revendication 6, dans lequel la séquence d'ADN codant pour une protéine ou un peptide d'intérêt est le gène MET-HSA fusionné à son extrémité 5' avec le promoteur hybride GAL/CYC1 ou GAL/PGK, et à son extrémité 3' avec un signal de terminaison de transcription qui fonctionne dans la levure.

8. Vecteur plasmidique 2 μ m selon l'une quelconque des revendications 1 à 4, dans lequel la séquence d'ADN codant pour une protéine ou un peptide d'intérêt est le gène *Saccharomyces cerevisiae* var. *diastaticus* DEX-1 ou une séquence d'ADN codant pour la β -glucanase de *Bacillus subtilis* fusionnée à son extrémité 5' avec un promoteur de gène qui fonctionne dans la levure par l'intermédiaire d'une séquence de contrôle de sécrétion qui fonctionne dans la levure, et à son extrémité 3' avec un signal de terminaison de transcription qui fonctionne dans la levure.

9. Vecteur plasmidique 2 μ m selon la revendication 1, ayant substantiellement la configuration de pSAC3 comme représenté sur la Figure 3 des dessins joints.

10. Vecteur plasmidique 2 μ m selon l'une quelconque des revendications 1 à 8, et portant un gène d'intérêt inséré directement ou indirectement sur le site *SnaBI*.

11. Procédé de préparation d'un vecteur plasmidique 2 μ m selon l'une quelconque des revendications précédentes, comprenant:

l'insertion dans un plasmide 2 μ m complet contenant une origine de réplication (ori) et un gène STB, (i) d'une séquence d'ADN pour sélectionner les transformants de levure, (ii) d'une séquence d'ADN codant pour une protéine ou un peptide d'intérêt, et (iii) d'un segment d'insertion comprenant (a) un ADN de plasmide bactérien permettant la propagation du vecteur dans les bactéries, et (b) les éléments d'un site de recombinaison FLP tels qu'un site supplémentaire de recombinaison FLP est créé dans le vecteur et ledit ADN bactérien est pris en sandwich entre deux sites de recombinaison

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FLP mutuellement en orientation directe, ladite séquence d'ADN qui code pour une protéine ou un peptide d'intérêt étant insérée entre ori et le gène STB de l'ADN dérivé de plasmide 2 μ m, de telle sorte que la transcription de ladite séquence d'ADN ne se continue pas à travers ori ou STB.

- 5 12. Procédé selon la revendication 11, dans lequel le segment d'insertion est inséré au site unique *Xba*I d'un site de recombinaison FLP endogène, une extrémité du segment d'insertion porte une partie d'une séquence répétée 2 μ m et l'autre extrémité du segment d'insertion porte le reste de la séquence répétée 2 μ m.
- 10 13. Cellule de levure contenant un plasmide 2 μ m ayant une origine de réplication (ori) et un gène STB et comprenant une séquence d'ADN non bactérien codant pour une protéine ou un peptide intéressant qui est hétérologue de la levure, le plasmide ne comprenant pas d'ADN bactérien qui a été transformé en ladite cellule, ladite séquence d'ADN qui code pour une protéine ou un peptide d'intérêt étant située entre ori et le gène STB, de telle sorte que la transcription de ladite séquence d'ADN ne se continue pas à travers ori ou STB.
- 15 14. Cellule de levure dérivée d'une cellule selon la revendication 13 par reproduction de cette cellule et par reproduction de sa descendance, et contenant un plasmide comprenant une séquence d'ADN codant pour une protéine ou un peptide d'intérêt qui est hétérologue à la levure, le plasmide ne comprenant pas d'ADN bactérien.
- 20 15. Levure de brasserie ou levure de laboratoire transformée par un vecteur plasmidique 2 μ m selon l'une quelconque des revendications 1 à 10 ou 14.
- 25 16. Procédé de préparation d'une protéine ou d'un peptide d'intérêt comprenant la fermentation d'une levure selon la revendication 13, 14 ou 15, et la séparation de la protéine ou du peptide d'intérêt.
17. Vecteur plasmidique de levure 2 μ m portant un gène d'intérêt inséré directement ou indirectement au site *Sna*BI.
- 30 18. Vecteur plasmidique 2 μ m comprenant une séquence d'ADN bactérien, trois sites de recombinaison FLP, dont une paire de sites est en orientation directe et les deux autres paires sont en orientation indirecte, et une séquence d'ADN codant pour une protéine ou un peptide d'intérêt, ladite séquence bactérienne étant située entre lesdits sites se trouvant en orientation directe de telle sorte que, quand une levure est transformée par ledit vecteur, ladite séquence bactérienne est éliminée, et la séquence d'ADN codant pour une protéine ou un peptide d'intérêt n'étant pas située entre lesdits sites se trouvant en orientation directe, ledit vecteur portant un gène d'intérêt inséré directement ou indirectement au site *Sna*BI.

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Fig. 1

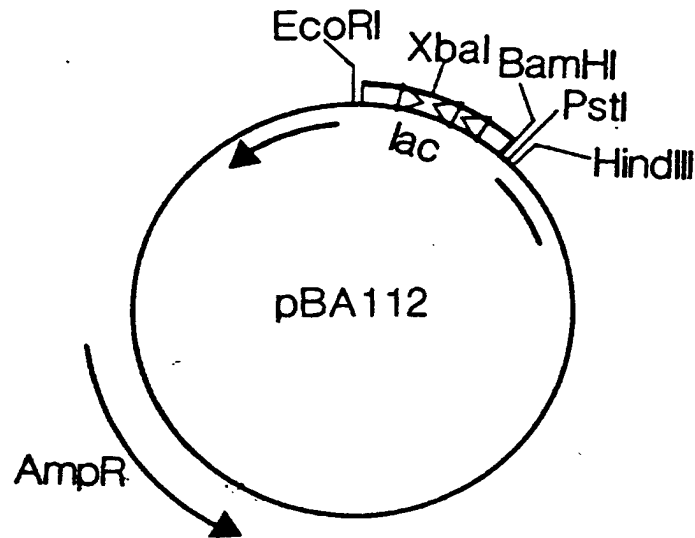
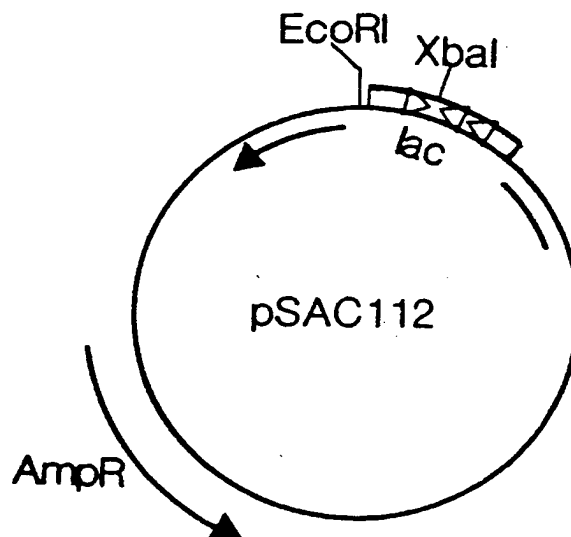
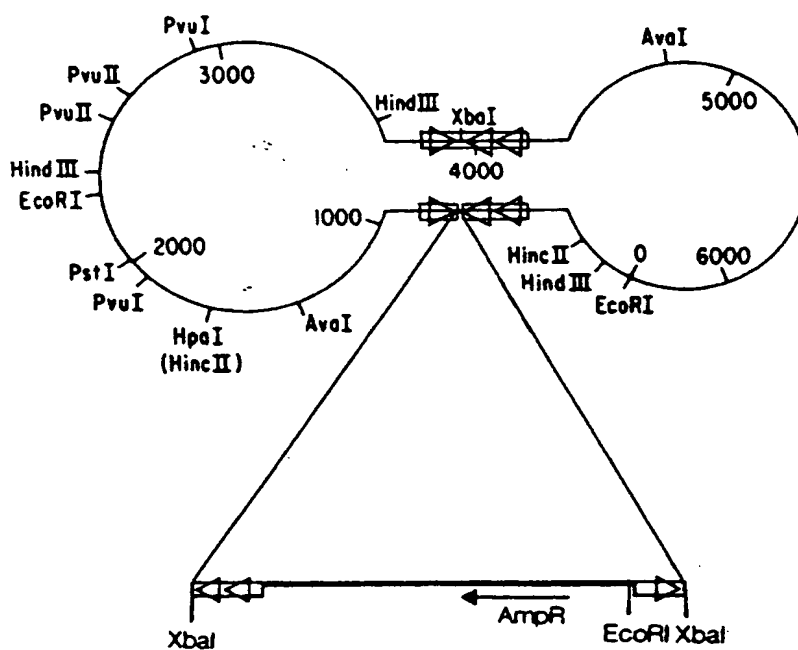


Fig. 2



g3



pSAC3

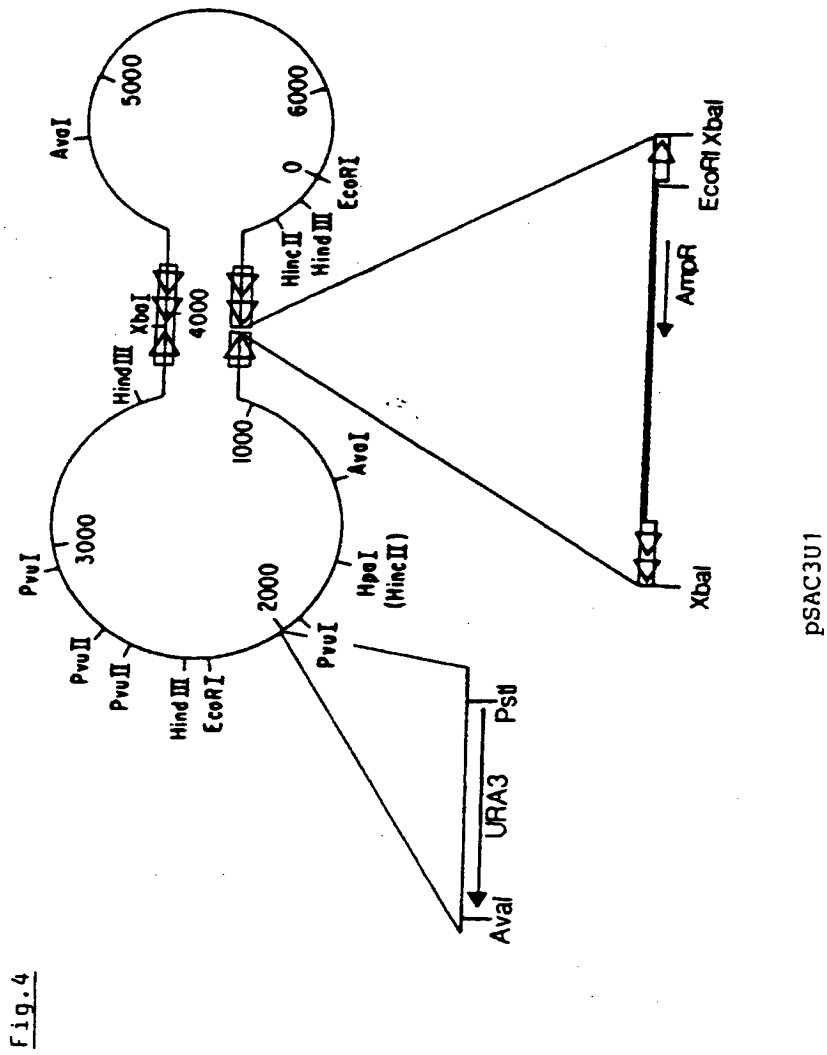


Fig. 5

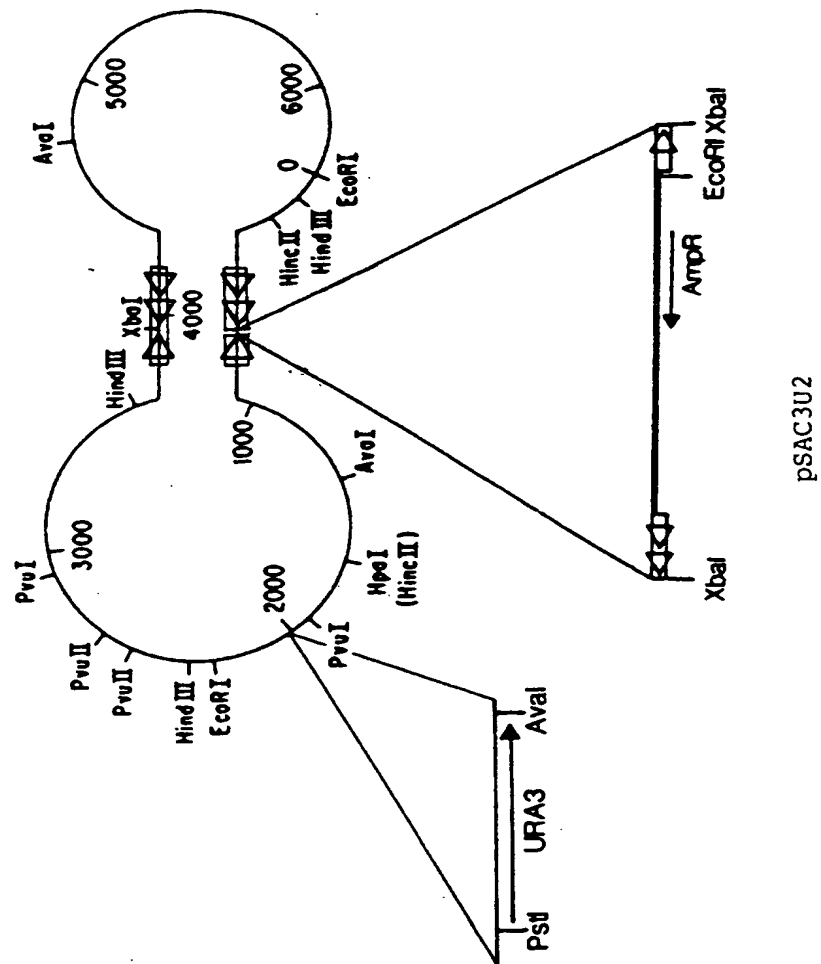
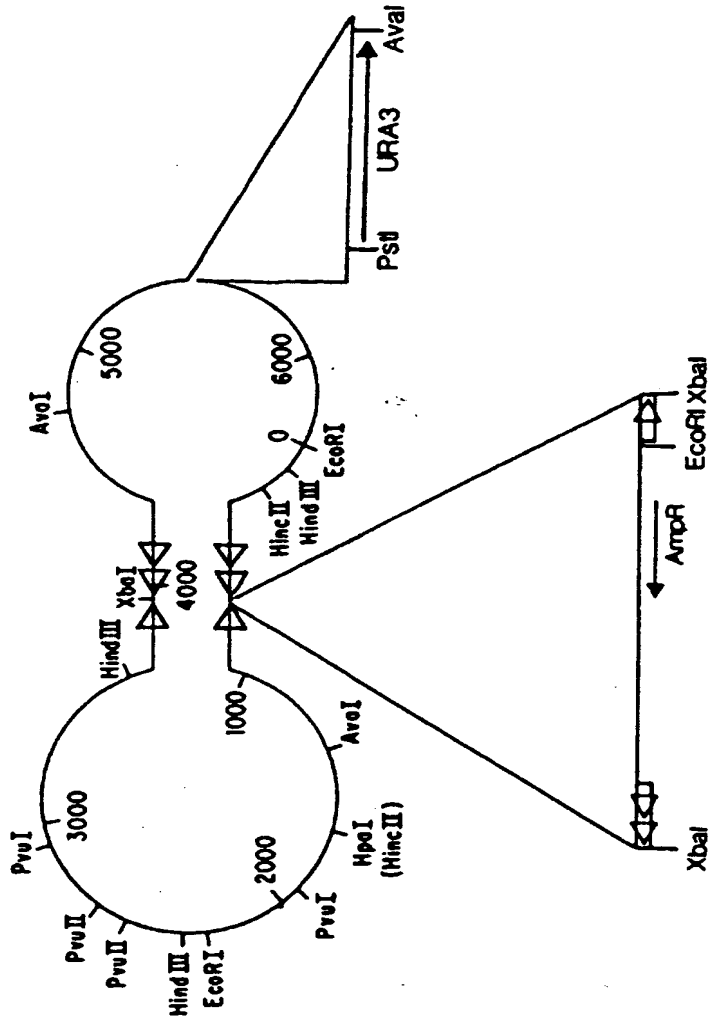
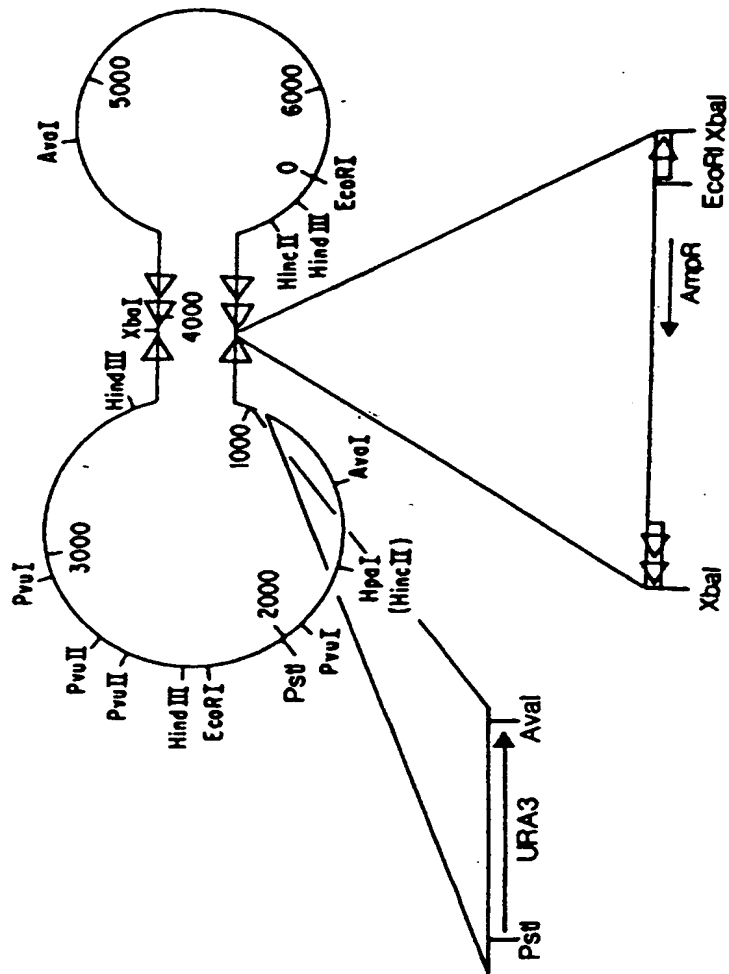


Fig. 6



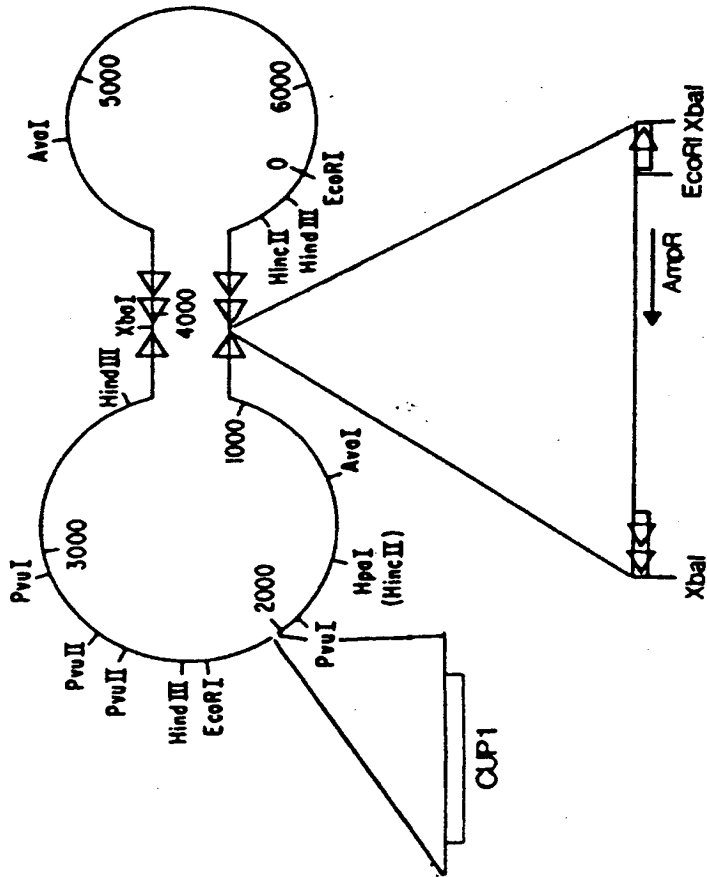
PSAC300

Fig. 7



PSAC310

Fig. 8



pSAC3C1

Figure 9.

Co-Inheritance of URA⁺ and bla⁺

